Published May 1, 2015

Identification of a novel microRNA important for melanogenesis in alpaca (*Vicugna pacos*)[1]

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ABSTRACT: The molecular mechanisms underlying the formation of coat colors in animals are poorly understood. Recent studies have demonstrated that microRNA play important roles in the control of melanogenesis and coat color in mammals. In a previous study, we characterized the miRNA expression profiles in alpaca skin with brown and white coat color and identified a novel miRNA (named lpa-miR-nov-66) that is expressed significantly higher in white skin compared to brown skin. The present study was conducted to determine the functional roles of this novel miRNA in the regulation of melanogenesis in alpaca melanocytes. lpa-miR-nov-66 is predicted to target the soluble guanylate cyclase (*sGC*) gene based on presence of a binding site in the sGC coding sequence (CDS). Overexpression of lpa-miR-nov-66 in alpaca melanocyes upregulated the expression of sGC both at the mRNA and protein level. Overexpression of lpa-miR-nov-66 in melanocyes also resulted in decreased expression of key melanogenic genes including tyrosinase (*TYR*), tyrosinase related protein 1 (*TYRP1*), and microphthalmia transcription factor (*MITF*). Our ELISA assays showed increased cyclic guanosine monophosphate (cGMP) but decreased cyclic adenosine monophosphate (cAMP) production in melanocytes overexpressing lpa-miR-nov-66. In addition, overexpression of lpa-miR-nov-66 also reduced melanin production in cultured melanocytes. Results support a role of lpa-miR-nov-66 in melanocytes by directly or indirectly targeting *sGC*, which regulates melanogenesis via the cAMP pathway.

Key words: lpa-miR-nov-66, melanin, melanocytes, soluble guanylate cyclase

J. Anim. Sci. 2015.93:1622–1631
doi:10.2527/jas2014-8404

INTRODUCTION

Coat color is an important production trait in fleece-producing animals, such as alpaca and sheep. The phenotype of coat color depends on 2 types of melanin (black to brown eumelanin and yellow to reddish brown pheomelanin) produced by melanocytes resident in the skin (Ito et al., 2000; Ito and Wakamatsu, 2008). The quality and ratio of eumelanin to pheomelanin dictate the coat color (Ito et al., 2000). The genetic basis for coat color is well documented in mice (Slominski et al., 2004; Steingrímsson et al., 2006), and many known genes controlling coat color in mice are also implicated in regulation of coat color in other species. For example, the loci of melanocortin-1 receptor (***MC1R***) and agouti signaling protein (***ASIP***) genes are functionally linked to undesirable coat color phenotypes in sheep (Våge et

[1]This work was supported by a grant from the National Natural Science Foundation of China (No.31201868), Science and technology key project of Shanxi (20120311024–2), and 863 project of China (No.2008021038).
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Received August 13, 2014.
Accepted January 29, 2015.

al., 1999; Norris and Whan, 2008). Tyrosinase-related protein 1 (***TYRP1***) is a strong positional candidate gene for color variation in Soay sheep (Gratten et al., 2007). The genes *MC1R* (Powell et al., 2008; Feeley and Munyard, 2009) and *ASIP* (Bathrachalam et al., 2012; Chandramohan et al., 2013) are associated with coat color of alpaca. A recent study demonstrated that solute carrier family 7 member 11 (***Slc7a11***) gene is expressed significantly higher in brown skin relative to white skin in alpaca (Tian et al., 2015). Despite considerable knowledge of the genetic regulation of coat color in mice and identification of loci involved in coat color regulation in fiber-producing species, the molecular and cellular mechanisms that regulate coat color in fiber-producing species are not completely understood.

MicroRNA (***miRNA***) are noncoding 21–25 nt RNA molecules that play an important role in regulating gene expression in development, physiology, and disease of animals (Bartel, 2004). Multiple studies on the expression and function of miRNA in the skin of mammalian species including mouse (Andl et al., 2006; Yi et al., 2006), goat and sheep (Wenguang et al., 2007), and alpaca (Zhu et al., 2010) have been reported. Tissue-specific miRNA, such as miR-203 in skin, have been documented (Sonkoly et al., 2007). Some miRNA are known to affect coat color phenotype. For example, overexpression of miR-137 in mice causes a change in skin color from black to brown (Dong et al., 2012).

To better understand the role of miRNA in the post-transcriptional regulation of genes linked to coat color of alpaca, we have characterized the miRNA in skin of alpaca with dark brown and white coat color by deep sequencing analysis (Tian et al., 2012) and identified a number of differentially expressed miRNA. In this study, we report the functional characterization of a novel miRNA (named lpa-miR-nov-66) that is expressed significantly higher in white alpaca skin compared to dark brown alpaca skin.

MATERIALS AND METHODS

Housing and care of alpaca and collection of skin samples were approved by the Animal Experimentation Ethics Committee of Shanxi Agricultural University, Taigu, China.

Construction of Plasmids

The lpa-miR-nov-66 expression plasmid was constructed by inserting an oligonucleotide corresponding to the sequence of the pre-lpa-miR-nov-66 into a mammalian expression vector, pcDNA6.2-GW/EmGFPmiR (Invitrogen, Carlsbad, CA), which contains a CMV promoter driving the expression of green fluorescent protein (**GFP**) and lpa-miR-nov-66. We have shown that this plasmid is capable of expressing mature lpa-miR-nov-66 and GFP simultaneously (data not shown). A negative control plasmid was also constructed using scrambled sequence of pre-lpa-miR-nov-66. The luciferase reporter plasmid was constructed by cloning the coding sequence of alpaca soluble guanylate cyclase (***sGC***) into a dual luciferase pmirGLO vector (Promega, Fitchburg, WI). A partial coding sequence of alpaca *sGC* containing the lpa-miR-nov-66 binding site was obtained by PCR using alpaca skin cDNA as a template with primers containing XbaI and NotI sites (Table 1). The PCR product and the vector were digested with XbaI and NotI and ligated together to obtain the pmirGLO-sGC-wt construct. The lpa-miR-nov-66 binding site in the sGC coding region of pmirGLO-sGC-wt was mutated using a site-directed gene mutagenesis kit (Beyotime, Shanghai, China) according to the manufacturer's instructions to obtain the pmirGLO-sGC-mut construct. All constructs were confirmed by sequencing.

Cell Culture and Transfection

All melanocyte cell cultures of alpaca used in this study were established by our lab and were maintained as previously described (Bai et al., 2010). Skin samples used for establishing the melanocyte lines were obtained from 3 white alpacas and 3 brown alpacas at the alpaca farm of Shanxi Agricultural University. Cells were transfected with the lpa-miR-nov-66 plasmid or the negative control plasmid using lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Three days after transfection, melanocytes were collected. Cell lysates and total RNA were prepared and subjected to Western blot and real-time PCR analyses, respectively.

Western Blot Analysis

Western blot analysis was performed as previously described (Dong et al., 2012) with the following primary antibodies: rabbit anti-sGC at 1:800 dilution (Abcam, Cambridge, UK), rabbit anti-microphthalmia transcription factor (**MITF**) at 1:200 dilution (Santa Cruz, CA), rabbit anti-tyrosinase (**TYR**) at 1:1000 dilution (Santa Cruz, CA), rabbit anti-TYRP1 at 1:1000 dilution (Abcam, Cambridge, UK), rabbit anti-β-actin (Sigma, St. Louis, MO). Anti-rabbit secondary antibody was purchased from Invitrogen. Immunoblots were scanned on a ChemiDOC XRS$^+$ imager (Bio-Rad, Hercules, CA) and protein levels were quantified using the Image-Pro Plus software (Olympus, Tokyo, Japan).

Table 1. Primers used in this study[1]

Primer name	Primer sequence 5'-3'	Application
sGC-F1	CAGCTGGAGAAGGAACTGGC	Real time PCR
sGC-R1	TGGCCCTCTGTGGTCAGTAG	Real time PCR
sGC-F2	CGAGCTCCAGATGATCTACTTGCCTGAA	RT-PCR
sGC-R2	GCTCTAGATATCTGAACAGATTCACCGTCTACT	RT-PCR
sGC-wt-F	CTAGTCTAGA CAGATGATCTACTTGCCTGAA	Luciferase reporter-wt
sGC-wt-R	ATAAGAATGCGGCCGCCTCGAGTATCTGAACAGATTCACCGTCTACT	Luciferase reporter-wt
sGC-mut-F	CGGGCATGAAGATACAGTTCCTCCTCAACGAC	Luciferase reporter-mut
sGC-mut-R	GTCGT TGAGGAGGAACTGTATCTTCATGCCCG	Luciferase reporter-mut
miR-66-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATCT	Real time PCR
miR-66-F	CGAGTGAATGGCACCTTAT	Real time PCR
Common-R	CGAGCAGTGCAGGGTCCGAGGT	Real time PCR
U6-RT	GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTCATCT	Real time PCR
U6-F	CTCGCTTCGGCAGCACA	Real time PCR
MITF-F	TCCCAAGTCAAATGATCCAG	Real time PCR
MITF-R	GAGCCTGCATTTCAAGTTCC	Real time PCR
TYR-F	GCTTTAGCAACTTCATGGGA	Real time PCR
TYR-R	CTTGTTCTTCTCTGGGACAC	Real time PCR
TYRP1-F	GCCTTCTTTCTCCCTTC	Real time PCR
TYRP1-R	CAGACCACTCGCCATT	Real time PCR
β-actin-F	CTAAGGAGAAGGGCCAGTCC	Real time PCR
β-actin-R	CTCAAGTTGGGGGACAAAAA	Real time PCR

[1]RT-PCR = reverse transcript PCR.

Immunocytochemistry

Melanocytes were washed 3 times in 0.1 M PBS for 3 min each, fixed in 4% paraformaldehyde, and then incubated at room temperature in 3% hydrogen peroxide for 15 min to block the action of any endogenous peroxidase. After washing with 0.1 M PBS 3 times for 5 min each, cells were immersed in BSA at 37°C for 40 min. Cells were then incubated at 4°C overnight in anti-sGC antibody solution. Following washing 3 times in 0.1 M PBS for 5 min each, cells were incubated with horseradish peroxidase (**HRP**)-conjugated anti-rabbit IgG for 30 min at 37°C. Immunostaining of the cells was then observed under a microscope (Leica). For negative controls, PBS was substituted for the primary antibody.

Quantitative Real-Time PCR for miRNA and mRNA

Total RNA from melanocytes was extracted using TRIzol reagent (Invitrogen) or an RNeasy kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions and treated with DNase I (Sigma). For mRNA quantification, 1 μg of total RNA was converted to cDNA using a cDNA synthesis kit (Takara, Dalian, China) according to the manufacturer's instructions, and quantitative real time PCR was performed using SYBR Green PCR master mix (Takara). For miRNA quantification, cDNA was generated using a cDNA synthesis kit (Takara), specific stem–loop RT primers, and a common reverse primer according to a previously established method for real-time quantification of miRNA (Chen. et al., 2005). Quantitative real time PCR was performed using SYBR Green PCR master mix (Takara). The primer sequences are listed in Table 1. Real-time PCR was performed on a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Melting curves for each sample were analyzed to validate specificity of amplification. All samples were run in triplicate and the relative amount of miRNA and mRNA was normalized to the amount of U6 and β-actin mRNA, respectively. Quantification of miRNA and mRNA transcript abundance was performed using the comparative threshold cycle ($\mathbf{C_T}$) method (Livak and Schmittgen, 2001). The difference in abundance of miRNA and target gene mRNA was determined by analysis of variance (SPSS 11.5 software, Chicago, IL).

cGMP and cAMP Assays

Following collection, cells (1×10^6) were treated with 0.1M HCl, incubated for 10 min, and visually inspected to verify cell lysis. The cell lysates were centrifuged at 600 × g for 10 min at room temperature and the supernatants were collected and used directly in the assays. Concentrations of cGMP and cAMP were determined by enzyme-linked immunosorbent assay after acetylation of the samples according to the manu-

facturer's instructions (NewEast Biosciences, King of Prussia, PA).

L-dopa Staining

L-dopa staining was performed following the established method (Bai et al., 2010). Melanocytes were washed with PBS twice before collection. Cells in 6-well plates were fixed with 10% formaldehyde, followed by washing with PBS twice. The cells were then stained by L-dopa for 5 h until pigmentation was observed.

Melanin Measurement

Measurement of melanin content was performed as previously described (Dong et al., 2011). In brief, melanocytes were harvested and rinsed with PBS followed by addition of l mL of 1N NaOH to dissolve the melanin. Melanin content was then measured spectrophotometrically at an absorbance of 475 nm. The melanin measurement was normalized to total amount of cells. All experiments were performed in triplicate.

Dual Luciferase Assay for miRNA Target Validation

For the luciferase reporter assay, 2 μg of pmirGLO-sGC-wt or pmirGLO-sGC-mut plasmid were cotransfected into 293T cells with lpa-miR-nov-66 plasmid or negative control miRNA plasmid using lipofectamine 2000 (Invitrogen). Luciferase activities in the transfected cells were measured with a dual-luciferase reporter assay kit (Promega) 2 d after cotransfection. Firefly luciferase activity was normalized to renilla luciferase activity to adjust for transfection efficiency.

Statistical Analysis

Data were analyzed using ANOVA and Fisher's protected LSD test. Data were reported as mean ± SE. The differences in abundance of target miRNA, mRNA, protein, cGMP, cAMP, and melanin production between control and experimental groups were determined by ANOVA using SPSS 11.5 software (SPSS, Chicago, IL).

RESULTS

Effect of lpa-miR-nov-66 Overexpression on mRNA and Protein Abundance of sGC

lpa-miR-nov-66 is a novel miRNA that was discovered by deep sequencing of miRNA in alpaca skin with white and dark brown color (Tian et al., 2012). Real time PCR analysis showed that the novel miRNA is expressed significantly higher in white skin compared to brown skin of alpaca (Fig. 1). The target genes of lpa-miR-nov-66 were predicted using miRanda software. One of the predicted targets of lpa-miR-nov-66 is soluble guanylate cyclase (*sGC*; Fig. 2). To determine whether *sGC* is a target of lpa-miR-nov-66, pre-miR-nov-66 was transfected into alpaca melanocytes to test the effect of lpa-miR-nov-66 on expression of *sGC*. Figure 3A shows enhanced accumulation of lpa-miR-nov-66 in melanocytes transfected with the miRNA expression plasmid compared to the negative control ($P < 0.001$; Fig. 3A). Overexpression of lpa-miR-nov-66 resulted in an increase in the abundance of both sGC mRNA (Fig. 3B) and protein (Fig. 3C and 3D), indicating that sGC is a potential target of lpa-miR-nov-66.

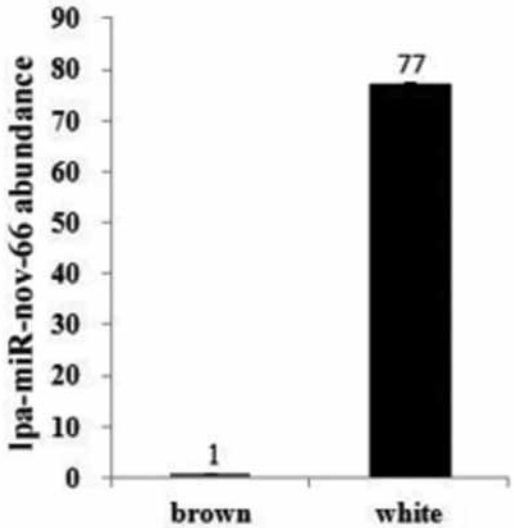

Figure 1. Real time PCR analysis of lpa-miR-nov-66 expression in white and brown alpaca skin. Data are expressed as mean ± SD ($n = 3$ animals/group).

Effect of lpa-miR-nov-66 Overexpression on the Expression of Melanogenic Genes

The abundance of both mRNA and protein for TYR, TYRP1, and MITF were examined in melanocytes transfected with the lpa-miR-nov-66 overexpression construct relative to cells transfected with the negative control plasmid. As shown in Fig. 4A, overexpression of lpa-miR-nov-66 in melanocytes did not affect *MITF* mRNA abundance but resulted in a decrease in the mRNA abundance for both *TYR* and *TYRP1*. Western blot analysis showed that the expression of TYR, TYRP1, and MITF proteins was significantly reduced in melanocytes overexpressing lpa-miR-nov-66 (Fig. 4B and 4C).

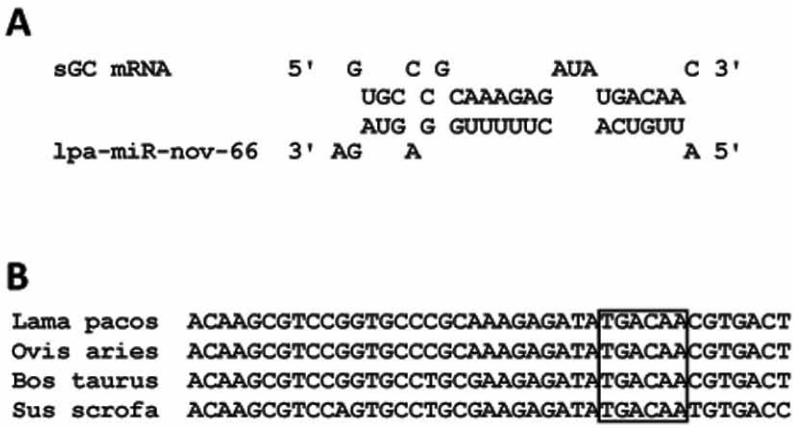

Figure 2. lpa-miR-nov-66 is predicted to target alpaca sGC gene. A. Predicted lpa-miR-nov-66 binding site in the coding sequence (CDS) of alpaca soluble guanylate cyclase (sGC) transcript. B. Alignment of sGC sequences around the miRNA binding site from cattle, sheep, pig and alpaca. The outlined boxes indicate the miRNA seed region.

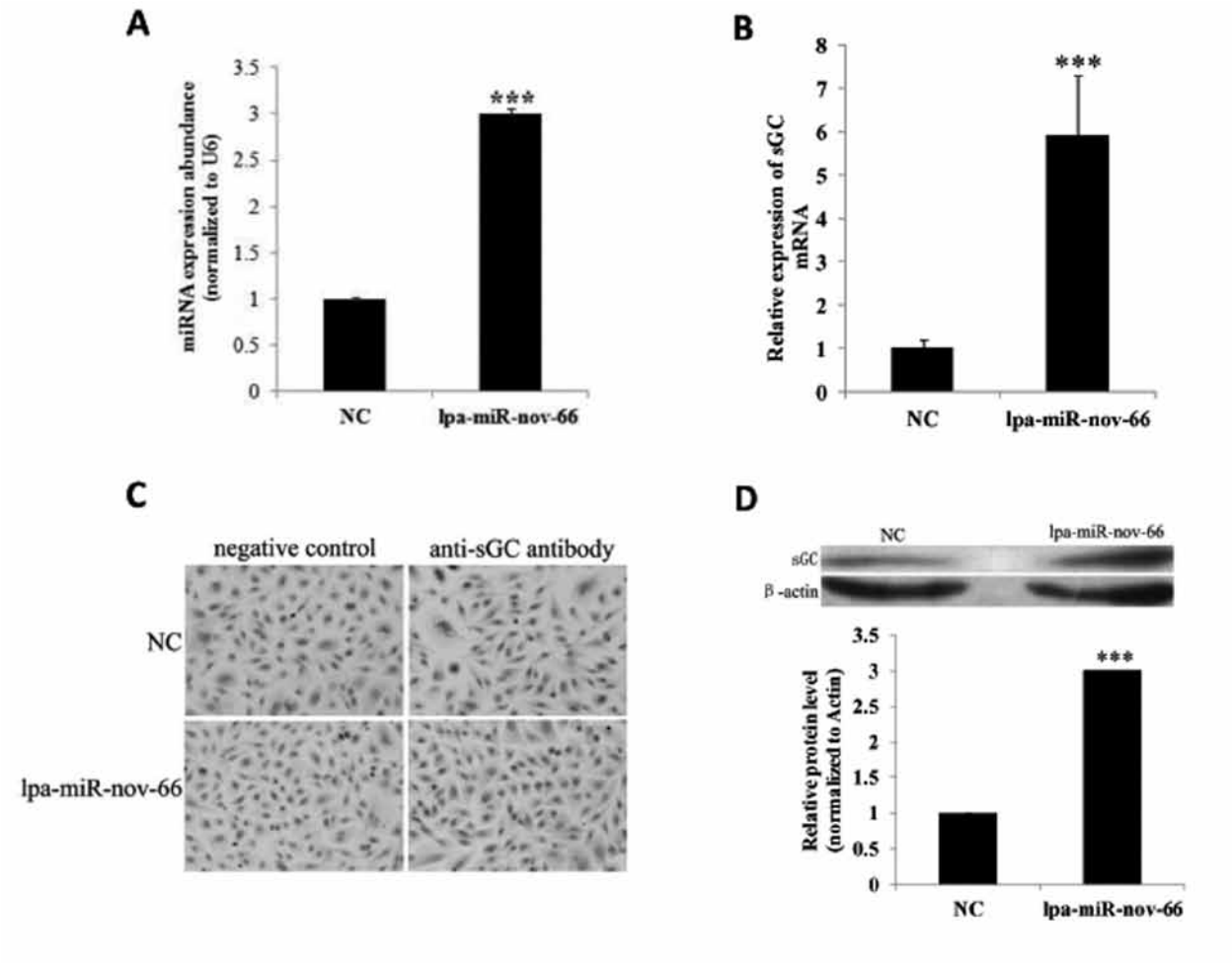

Figure 3. Effect of lpa-miR-nov-66 on mRNA and protein abundance of soluble guanylate cyclase (sGC) . (A) Real time PCR analysis of lpa-miR-nov-66 expression in melanocytes transfected with the lpa-miR-nov-66 expression plasmid. Data are expressed as mean ± SD from 3 replicates. (B) Real time PCR analysis of alpaca sGC expression in melanocytes transfected with the lpa-miR-nov-66 expression plasmid. Data are expressed as mean ± SD from 3 replicates. (C) and (D) Analysis of sGC protein expression in melanocytes transfected with the lpa-miR-nov-66 expression plasmid using immunocytochemical and western blot detection, respectively. *** $P < 0.001$.

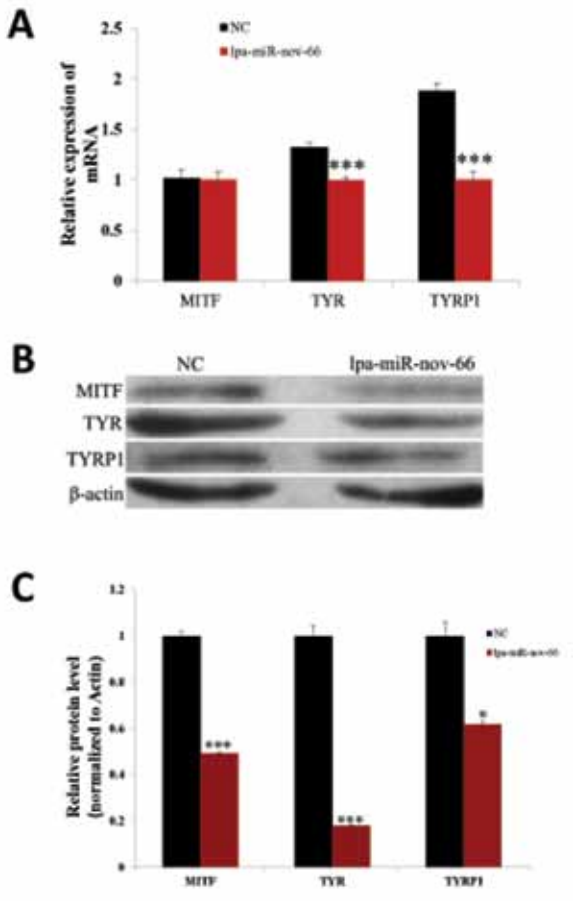

Figure 4. Effect of lpa-miR-nov-66 on the expression of coat color genes in melanocytes. (A) Real time PCR analysis of microphthalmia transcription factor (MITF), tyrosinase (TYR), and tyrosinase related protein 1 (TYRP1) mRNA expression in melanocytes transfected with the lpa-miR-nov-66 expression plasmid. Data are expressed as mean ± SD from 3 replicates. (B) Western blot analysis of MITF, TYR, and TYRP1 protein expression in melanocytes transfected with the lpa-miR-nov-66 expression plasmid. (C) Densitometric analysis of MITF, TYR ,and TYRP1 protein expression using the Image-Pro Plus software (Olympus, Tokyo, Japan). Data were normalized to β-actin and expressed as relative fold change (mean ± SD, $n = 3$). * $P < 0.05$; *** $P < 0.001$.

Effect of lpa-miR-nov-66 Overexpression on the Production of cGMP and cAMP

The production of cGMP was increased by 2.05-fold in melanocytes transfected with lpa-miR-nov-66 plasmid (Fig. 5A), whereas overexpression of lpa-miR-nov-66 in melanocytes resulted in a decrease in cAMP production with 0.57-fold change as shown by ELISA analysis (Fig. 5B).

Effect of lpa-miR-nov-66 Overexpression on Melanin Production

L-dopa staining for tyrosinase activity showed that melanocytes transfected with lpa-miR-nov-66 plasmid were melanogenic. The amount of L-dopa was less in the lpa-miR-nov-66 transfected cells than in the negative control transfected cells (Fig. 6A). Overexpression of lpa-miR-nov-66 in melanocytes reduced melanin production by 30% (Fig. 6B).

lpa-miR-nov-66 Targets the Predicted miRNA Binding Site in the Coding Sequence of sGC

To validate the specificity of lpa-miR-nov-66 regulation of *sGC* through the predicted miRNA binding site in the coding sequence (**CDS**) of *sGC*, luciferase reporter assays were performed using luciferase reporter constructs containing either the wild-type sGC CDS (pmirGLO-sGC-wt) or the mutant sGC CDS (pmirGLO-sGC-mut). The constructs were cotransfected into 293T cell with the lpa-miR-nov-66 expression plasmid or negative control plasmid. Dual luciferase reporter assays showed that the reporter activity in cells cotransfected with pmirGLO-sGC-wt and lpa-miR-nov-66 plasmid was decreased by 30% compared with the cells cotransfected with pmirGLO-sGC-wt and the negative control plasmid (Fig. 7A). Luciferase reporter activity in cells cotransfected with pmirGLO-sGC-mut and lpa-miR-nov-66 plasmid was similar to the reporter activity in cells cotransfected with pmirGLO-sGC-mut and the negative control miRNA plasmid (Fig. 7B). These data indicate that lpa-miR-nov-66 can bind and regulate sGG in sequence specific fashion through the predicted binding site in the CDS.

DISCUSSION

Melanocytes are specialized cells in which melanin synthesis (eumelanins and pheomelanins) takes place. Melanin is responsible for skin and hair color in mammalian species. During the process of determination of skin or hair color, many precise mechanisms play important roles in governing their regulation. In recent years, we have been focusing our attention on the molecular mechanisms of regulating melanogenesis in fiber-producing animals, especially in alpaca. miRNA play a key role in animal pigmentation. Several miRNA, including miR-340, miR-137, miR-182, and miR-25, were shown to regulate pigmentation by targeting *MITF* mRNA (Bemis et al., 2008; Segura et al., 2009; Goswami et al., 2010; Zhu et al., 2010). Our recent study demonstrated that coat color was regulated by miR-137 targeting *MITF* in transgenic mice (Dong et al., 2012).

From deep sequencing analysis of miRNA in alpaca skin with white and brown coat color, we have obtained 15 and 18 potentially novel miRNA from white and brown alpaca skin, respectively (Tian et al., 2012). lpa-miR-nov-66 is one of the novel miRNA that shows significantly higher expression in white vs. brown skin of alpaca. *sGC* is one of the predicted

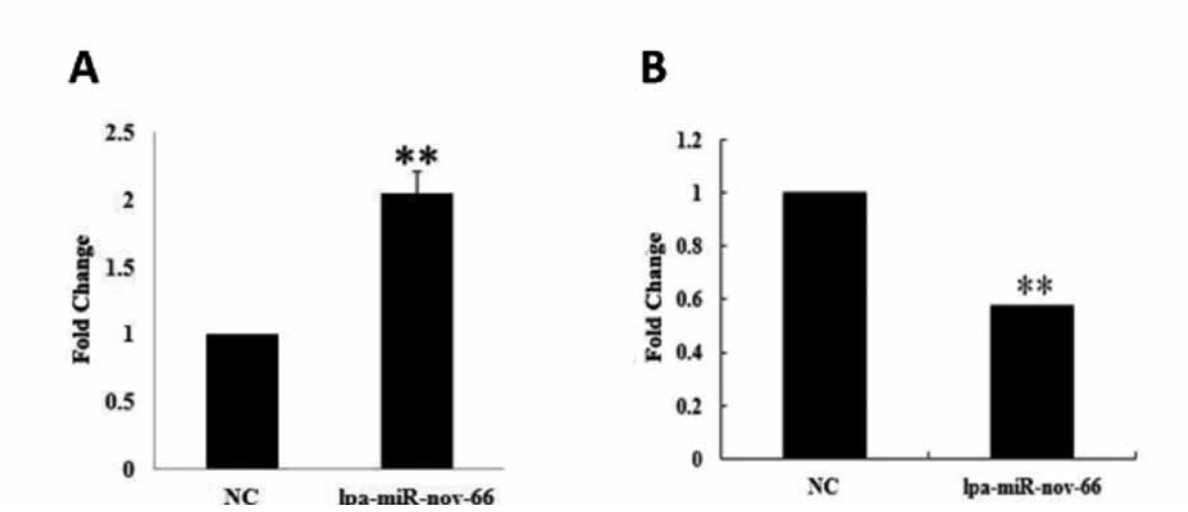

Figure 5. Effect of lpa-miR-nov-66 on the production of cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) in melanocytes. (A) Amount of cGMP in melanocytes transfected with lpa-miR-nov-66 plasmid. (B) Amount of cAMP in melanocytes overexpressing lpa-miR-nov-66. ** $P < 0.01$.

target genes for lpa-miR-nov-66. Overexpression of lpa-miR-nov-66 in alpaca melanocytes upregulates the expression of sGC both at the transcriptional and translational level (Fig. 3). To our knowledge, upregulation of target genes by miRNA binding to the CDS of target genes has not been reported before. In general, the actions of miRNA are through degradation of mRNA or translational suppression of target genes by binding to the 3' untranslated regions (**UTR**) of target mRNA (Bartel, 2004; Wu et al., 2006). Downregulation of gene expression by miRNA binding to the CDS of target mRNA has also been reported (Forman et al., 2008; Wang et al., 2010; Schnall-Levin et al., 2011). Genes with shorter 3'UTR are favorably targeted in the CDS by miRNA (Reczko et al., 2012) and binding sites located in the CDS are more effective in inhibiting translation than sites located in the 3'UTR (Hausser et al., 2013). Reports of upregulation of target genes by miRNA are limited. A few studies have demonstrated that miRNA can stabilize mRNA and enhance gene expression by binding to the 5'UTR of target mRNA (Ørom et al., 2008; Tsai et al., 2009). The observed effect of lpa-miR-nov-66 resulting in upregulation of *sGC* expression may represent a new mechanism of miRNA action. However, we cannot rule out the possibility that *sGC* expression is regulated indirectly by lpa-miR-nov-66. We have attempted to determine if sGC is a direct target of lpa-miR-nov-66 by luciferase reporter gene assays. The results showed that overexpression of lpa-miR-nov-66 in 293T cells decreased the luciferase reporter activity, which contradicts the effect of lpa-miR-nov-66 on *sGC* expression (upregulation). Luciferase reporter gene assays typically insert 3'UTR containing predicted binding sites in luciferase construct and hence it is not clear whether insertion of a partial CDS of *sGC* containing the miRNA binding

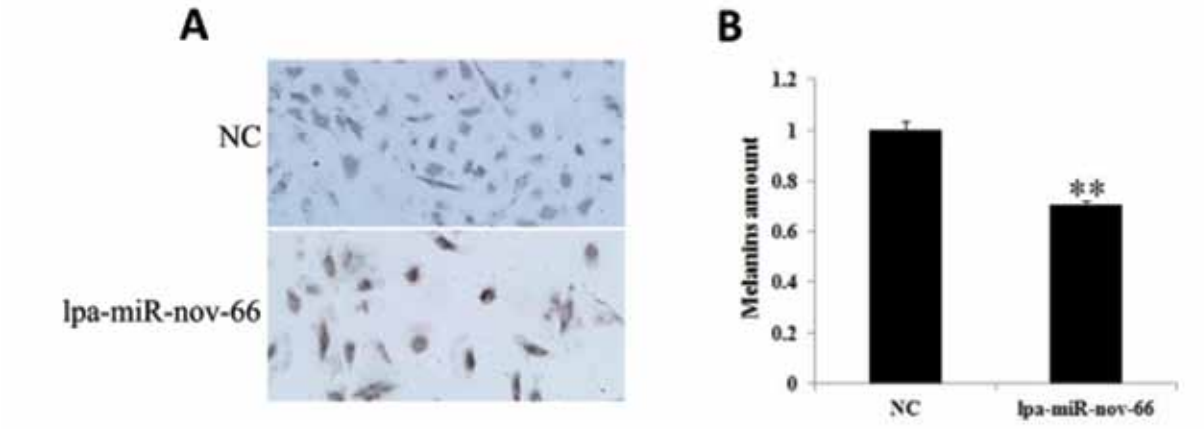

Figure 6. Effect of lpa-miR-nov-66 on melanin production. (A) L-dopa staining of melanocytes transfected with lpa-miR-nov-66 expression plasmid (lower panel) and negative control plasmid (NC, upper panel). (B) Melanin production in melanocytes overexpressing lpa-miR-nov-66. Data are expressed as mean ± SD from 3 replicates. **$P < 0.01$.

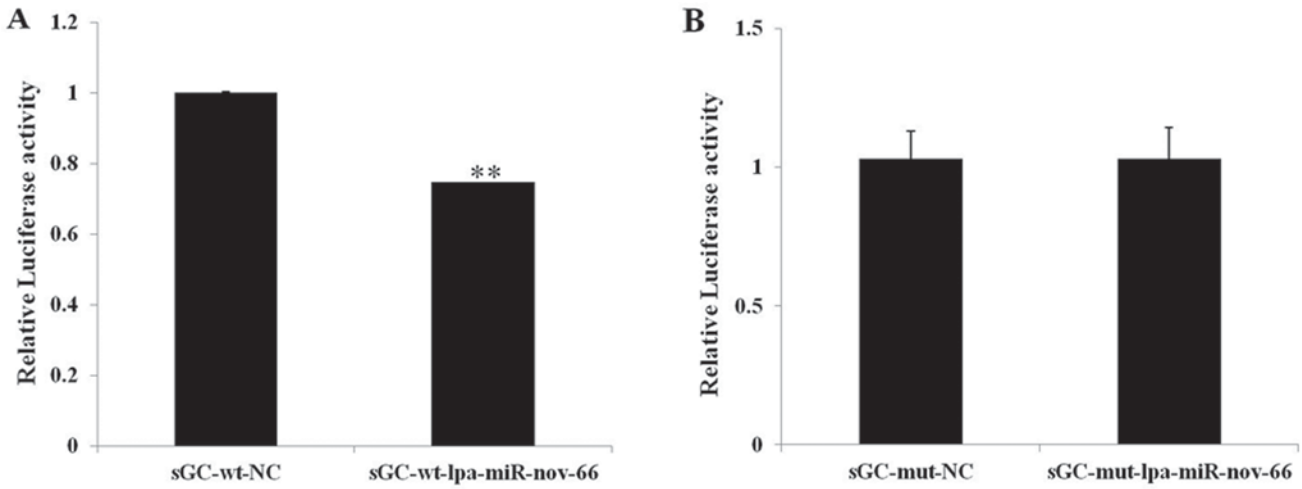

Figure 7. Luciferase reporter assays to determine the specificity of lpa-miR-nov-66 binding site in the coding sequence (CDS) of soluble guanylate cyclase (sGC) transcript. (A) Luciferase reporter activities in 293T cells cotransfected with the reporter construct containing the wild-type sGC CDS (pmirGLO-sGC-wt) and the lpa-miR-nov-66 or the negative control plasmid (NC). (B) Luciferase reporter activities in 293T cells cotransfected with the mutant reporter construct (pmirGLO-sGC-mut) and the lpa-miR-nov-66 or NC. The Firefly luciferase activities were normalized to the Renilla luciferase activities. Data are expressed as relative luciferase activities (n = 3, mean ± SEM).

site within 3'UTR of the construct would appropriately mimic regulation of endogenous sGC. Further investigations are needed to determine how exactly *sGC* is regulated by lpa-miR-nov-66.

sGC catalyzes the conversion of guanosine triphosphate (**GTP**) to cGMP in response to various extracellular stimuli, thereby providing an important second messenger for the regulation of protein kinase, phosphodiesterases (**PDE**) and ion channels (Zabel et al., 1998). cGMP is capable of modulating cAMP levels through cGTP-regulated PDE (Stangherlin et al., 2011). The molecular mechanisms involved in the cAMP-regulation of cellular functions in melanocytes have been well studied (Buscà and Ballotti, 2000; Tachibana 2000). Our recent study of the transcriptome profiles in sheep skin showed that sGC was expressed differentially in animals with white versus black coat color (Fan et al., 2013). Differential expression of sGC in skin of alpacas with white and brown coat color has also been observed (supplementary Supporting Information). Therefore, lpa-miR-nov-66 may play a role in melanogenesis in alpaca melanocytes by regulating *sGC*, which potentially is a coat-color gene.

In this study, we show that overexpression of lpa-miR-nov-66 in alpaca melanocytes in vitro upregulates cGMP but downregulates cAMP production. In melanocytes, cAMP activates several intracellular signaling pathways that are involved in the regulation of pigmentation (Buscà and Ballotti, 2000). cAMP binds the 2 sites of the regulatory subunit of protein kinase A (**PKA**), allowing the catalytic subunit to be liberated and activated (Roesler et al., 1998). cAMP activates PKA, and PKA phosphorylates and activates cAMP responsive element binding protein (**CREB**), which, when activated, binds to the cAMP responsive element (**CRE**) domain present in the MITF promoter, thereby upregulating its transcription (Buscà and Ballotti, 2000).

PKA also phosphorylates the nuclear protein, CREB-binding protein (**CBP**), interaction of which with the CREB family proteins is required for MITF expression in normal human melanocytes. MITF itself is a transcription factor and can specifically bind M box and E box motifs and to upregulate TYR, TYRP1, and tyrosinase related protein 2 (**TYRP2**) promoter activities (Bertolotto et al., 1998). Given the important role of the above genes in promoting melanogenesis, the observed decrease in melanin production in melanocytes overexpressing lpa-miR-nov-66 is likely linked to observed decrease in cAMP. The proposed pathway for lpa-miR-nov-66 regulation of melanogenesis in alpaca melanocytes is illustrated in Fig. 8.

In conclusion, we provided evidence supporting an important functional role for lpa-miR-nov-66 in alpaca melanocytes by possibly targeting CDS of *sGC*, which regulates melanogenesis via the cAMP pathway. The post-transcriptional expression of MITF, TYR, and TYRP1 is decreased consequently, thereby resulting in decreased melanin production. This study revealed a functional role for a novel miRNA in regulation of melanogenesis and hence a potential candidate gene for producing natural coat colors in fiber-producing species by transgenic approach.

LITERATURE CITED

Andl, T., E. P. Murchison, F. Liu, Y. Zhang, M. Yunta-Gonzalez, J. W. Tobias, C. D. Andl, J. T. Seykora, G. J. Hannon, and S. E. Millar. 2006. The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. Curr. Biol. 16:1041–1049.

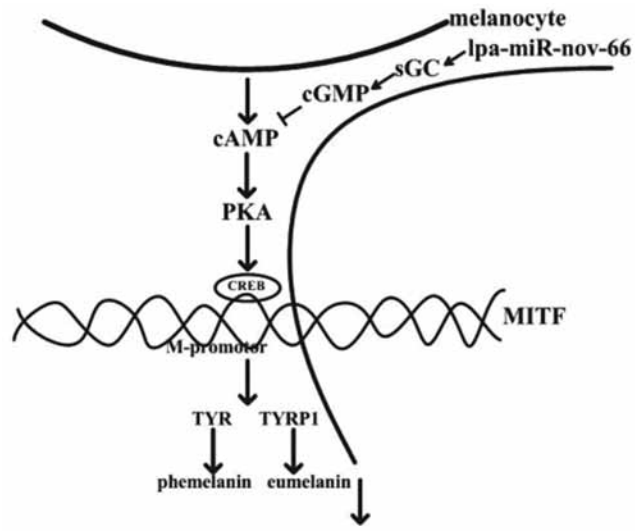

Figure 8. Potential pathway of lpa-miR-nov-66 in regulating melanogenesis in melanocytes of alpaca.

Bai, R., A. Sen, Z. Yu, G. Yang, H. Wang, R. Fan, L. Lv, K.-B. Lee, G. W. Smith, and C. Dong. 2010. Validation of methods for isolation and culture of alpaca melanocytes: A novel tool for in vitro studies of mechanisms controlling coat color. Asian Aust J Anim Sci 23:430–436.

Bartel, D. P. 2004. MicroRNAs: Genomics, biogenesis, mechanism, and function. Cell 116:281–297.

Bathrachalam C., Manna V. L., Renieri C., Terza A. L. 2012. Asip and MCIR cDNA polymorphism in alpaca.Fibre production in South American camelids and other fibre animals. Wageningen Academic Publishers, Wageningen, the Netherlands. p. 93–96.

Bemis, L. T., R. Chen, C. M. Amato, E. H. Classen, S. E. Robinson, D. G. Coffey, P. F. Erickson, Y. G. Shellman, and W. A. Robinson. 2008. MicroRNA-137 targets microphthalmia-associated transcription factor in melanoma cell lines. Cancer Res. 68:1362–1368.

Bertolotto, C., R. Buscà, P. Abbe, K. Bille, E. Aberdam, J. P. Ortonne, and R. Ballotti. 1998. Different cis-acting elements are involved in the regulation of TRP1 and TRP2 promoter activities by cyclic AMP: Pivotal role of M boxes (GTCATGTGCT) and of microphthalmia. Mol. Cell. Biol. 18:694–702.

Buscà, R., and R. Ballotti. 2000. Cyclic AMP a key messenger in the regulation of skin pigmentation. Pigment Cell Res. 13:60–69.

Chandramohan, B., C. Renieri, V. La Manna, and A. La Terza. 2013. The alpaca agouti gene: Genomic locus, transcripts and causative mutations of eumelanic and pheomelanic coat color. Gene 521:303–310.

Chen C., D. A. Ridzon, A. J. Broomer, Z. Zhou, D. H. Lee, J. T. Nguyen, M. Barbisin, N. L. Xu, V. R. Mahuvakar, M. R. Anderson, K. Q. Lao, K. J. Livak, and K. J. Guegler. 2005. Real-time quantification of microRNAs by stem-loop RT-PCR. Nucleic Acids Res. 33(20):e179.

Dong, C., H. Wang, L. Xue, Y. Dong, L. Yang, R. Fan, X. Yu, X. Tian, S. Ma, and G. W. Smith. 2012. Coat color determination by miR-137 mediated down-regulation of microphthalmia-associated transcription factor in a mouse model. RNA 18:1679–1686.

Dong, Y., H. Wang, J. Cao, J. Ren, R. Fan, X. He, G. W. Smith, and C. Dong. 2011. Nitric oxide enhances melanogenesis of alpaca skin melanocytes in vitro by activating the MITF phosphorylation. Mol. Cell. Biochem. 352:255–260.

Fan, R., J. Xie, J. Bai, H. Wang, X. Tian, R. Bai, X. Jia, L. Yang, Y. Song, M. Herrid, W. Gao, X. He, J. Yao, G. W. Smith, and C. Dong. 2013. Skin transcriptome profiles associated with coat color in sheep. BMC genomics. 14:389–400.

Feeley, N. L., and K. A. Munyard. 2009. Characterisation of the melanocortin 1 receptor gene in alpaca and identification of possible markers associated with phenotypic variations. Anim. Prod. Sci. 49:675–681.

Forman, J. J., A. Legesse-Miller, and H. A. Coller. 2008. A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. Proc. Natl. Acad. Sci. USA 105:14879–14884.

Gratten, J., D. Beraldi, B. V. Lowder, A. F. McRae, P. M. Visscher, J. M. Pemberton, and J. Slate. 2007. Compelling evidence that a single nucleotide substitution in *TYRP1* is responsible for coat-colour polymorphism in a free-living population of Soay sheep. Proceedings of the Royal Society B: Biological 274(1610):619–626.

Goswami, S., R. S. Tarapore, J. J. Teslaa, Y. Grinblat, V. Setaluri, and V. S. Spiegelman. 2010. MicroRNA-340-mediated degradation of microphthalmia-associated transcription factor mRNA is inhibited by the coding region determinant-binding protein. J. Biol. Chem. 285:20532–20540.

Hausser, J., A. P. Syed, B. Bilen, and M. Zavolan. 2013. Analysis of CDS-located miRNA target sites suggests that they can effectively inhibit translation. Genome Res. 23:604–615.

Ito, T., K. Tashiro, S. Muta, R. Ozawa, T. Chiba, M. Nishizawa, K. Yamamoto, S. Kuhara, and Y. Sakaki. 2000. Toward a protein-protein interaction map of the budding yeast: A comprehensive system to examine two-hybrid interactions in all possible combinations between the yeast proteins. Proc. Natl. Acad. Sci. USA 97:1143–1147.

Ito, S., and K. Wakamatsu. 2008. Chemistry of mixed melanogenesis-pivotal roles of dopaquinone. Photochem. Photobiol. 84:582–592.

Livak, K. J., and T. D. Schmittgen. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method. Methods 25:402–408.

Norris, B. J., and V. A.Whan. 2008. A gene duplication affecting expression of the ovine ASIP gene is responsible for white and black sheep. Genome Res. 18:1282–1293.

Ørom, U. A., F. C. Nielsen, and A. H. Lund. 2008. MicroRNA-10a binds the 5′ UTR of ribosomal protein mRNAs and enhances their translation. Mol. Cell 30:460–471.

Powell, A. J., M. J. Moss, L. T. Tree, B. L. Roeder, C. L. Carleton, E. Campbell, and D. L. Kooyman. 2008. Characterization of the effect of Melanocortin 1 Receptor, a member of the hair color genetic locus, in alpaca (*Lama pacos*) fleece color differentiation. Small Rumin. Res. 79:183–187.

Reczko, M., M. Maragkakis, P. Alexiou, I. Grosse, and A. G. Hatzigeorgiou. 2012. Functional microRNA targets in protein coding sequences. Bioinformatics 28:771–776.

Roesler, W. J., E. A. Park, and P. J. McFie. 1998. Characterization of CCAAT/enhancer-binding protein as a cyclic AMP-responsive nu-clear regulator. J. Biol. Chem. 273:14950–14957.

Schnall-Levin, M., O. S. Rissland, W. K. Johnston, N. Perrimon, D. P. Bartel, and B. Berger. 2011. Unusually effective microRNA targeting within repeat-rich coding regions of mammalian mRNAs. Genome Res. 21:1395–1403.

Segura, M. F., D. Hanniford, S. Menendez, L. Reavie, X. Zou, S. Alvarez-Diaz, J. Zakrzewski, E. Blochin, A. Rose, D. Boqunovic, D. Polsky, J. Wei, P. Lee, I. Belitskaya-Levy, N. Bhardwaj, I. Osman, and E. Hernando. 2009. Aberrant miR-182 expression promotes melanoma metastasis by repressing FOXO3 and microphthalmia-associated transcription factor. Proc. Natl. Acad. Sci. USA 106:1814–1819.

Slominski, A., D. J. Tobin, S. Shibahara, and J. Wortsman. 2004. Melanin pigmentation in mammalian skin and its hormonal regulation. Physiol. Rev. 84:1155–1228.

Sonkoly, E., T. Wei, P. C. J. Janson, A. Sääf, L. Lundeberg, M. Tengvall-Linder, G. Norstedt, H. Alenius, B. Homey, A. Scheynius, M. Ståhle, and Andor Pivarcsi. 2007. MicroRNAs: Novel regulators involved in the pathogenesis of psoriasis? PLoS ONE 2:E610.

Stangherlin, A., F. Gesellchen, A. Zoccarato, A. Terrin, L.A. Fields, M. Berrera, N. C. Surdo, M. A. Craig, G. Smith, G. Hamilton, and M. Zaccolo. 2011. cGMP signals modulate cAMP levels in a compartment-specific manner to regulate catecholamine-dependent signaling in cardiac myocytes. Circ. Res. 108:929–939.

Steingrímsson E., N. G. Copeland, and N. A. Jenkins. 2006. Mouse coat color mutations: From fancy mice to functional genomics. Dev. Dyn. 235:2401–2411.

Tachibana, M. 2000. MITF: A stream flowing for pigment cells. Pigment Cell Res. 13:230–240.

Tian, X., J. Jiang, R. Fan, H. Wang, X. Meng, X. He, J. He, H. Li, J. Geng, X. Yu, Y. Song, D. Zhang, J. Yao, G. W. Smith, and C. Dong. 2012. Identification and characterization of microRNAs in white and brown alpaca skin. BMC Genomics 13:555.

Tian, X., X. Meng, L. Wang, Y. Song, D. Zhang, Y. Ji, X. Li, and C. Dong. 2015. Molecular cloning, mRNA expression and tissue distribution analysis of Slc7a11 gene in alpaca (Lama paco) skins associated with different coat colors. Gene. 555:88–94.

Tsai, N., Y. Lin, and L. Wei. 2009. MicroRNA mir-346 targets the 5prime-untranslated region of receptor-interacting protein 140 (RIP140) mRNA and up-regulates its protein expression. Biochem. J. 424:411–418.

Våge, D. I., H. Klungland, D. Lu, and R. D. Cone. 1999. Molecular and pharmacological characterization of dominant black coat color in sheep. Mamm. Genome 10:39–43.

Wang, W.-X., B. R. Wilfred, K. Xie, M. H. Jennings, Y. Hu, A. J. Stromberg, and P. Nelson. 2010. Individual microRNAs (miRNAs) display distinct mRNA targeting "rules". RNA Biol. 7:373–380.

Wenguang, Z., W. Jianghong, L. Jinquan, and M. Yashizawa. 2007. A subset of skin-expressed microRNAs with possible roles in goat and sheep hair growth based on expression profiling of mammalian microRNAs. OMICS 11:385–396.

Wu, L., J. Fan, and J. G. Belasco. 2006. MicroRNAs direct rapid deadenylation of mRNA. Proc. Natl. Acad. Sci. USA 103:4034–4039.

Yi, R., D. O'Carroll, H. A. Pasolli, Z. Zhang, F. S. Dietrich, A. Tarakhovsky, and E. Fuchs. 2006. Morphogenesis in skin is governed by discrete sets of differentially expressed microRNAs. Nat. Genet. 38:356–362.

Zabel, U., M. Weeger, and H. Scnmidt. 1998. Human soluble guanylate cyclase: Functional expression and revised isoenzyme family. Biochem. J. 335:51–57.

Zhu, Z., J. He, X. Jia, J. Jiang, R. Bai, X. Yu, L. Lv, R. Fan, X. He, J. Geng, R. You, Y. Dong, D. Qiao, K. B. Lee, G. W. Smith, and C. Dong. 2010. MicroRNA-25 functions in regulation of pigmentation by targeting the transcription factor MITF in alpaca (*Lama pacos*) skin melanocytes. Domest. Anim. Endocrinol. 38:200–209.